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14. ABSTRACT

Dissemination and metastasis of tumor cells are major causes of morbidity and mortality in breast cancer patients. Therefore it is of vital importance to identify druggable targets to inhibit breast tumor invasion and metastasis. A critical component in the invasive growth, dissemination, and metastasis of cancer is acquisition of motility by tumor cells. Our preliminary studies suggest a novel role for the thromboxane A2 receptor (TP) in controlling breast tumor cell motility via regulating cytoskeleton reorganization. The objective of this proposal is to define the function of TP in tumor cell motility and to validate TP as a target for anti-metastasis therapy of breast cancer. In the first aim, the role of TP in breast tumor cell motility will be determined in the presence or absence of TP activation or inhibition. The isoform(s) of TP involved in tumor cell contraction and motility will be identified in the second specific aim. In the third aim, an orthotopic mouse model will be used to assess whether TP can be targeted to reduce breast cancer metastasis. The proposed studies will be highly significant toward the goals of developing mechanism-based interventions for cancers.

15. SUBJECT TERMS

Breast cancer, metastasis, thromboxane, G protein coupled receptor, migration, invasion

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Introduction

Major causes of death in breast cancer patients are the spread and metastasis of tumors. “Metastasis” means that the cancer cells have detached from the original tumor site and started to grow in another part of the body. The goal of this research project is to find out how breast cancer cells disseminate and move so that new methods can be developed to identify and treat breast cancer. For a tumor cell to move, it must push forward in the front and contract in the rear to power ahead. The main idea of the proposed project is that tumor dissemination and migration can be inhibited by blocking tumor cell contraction. Our laboratory has identified a cell receptor (TP) that receives information (“receptor”) in breast tumors and then participates in cell contraction and migration. This receptor can be activated by a lipid called thomboxane $A_{2.}$ A published study suggests that the level of this receptor expressed in breast tumor tissues have been linked with poor prognosis and a significant decrease in disease free survival. Our preliminary studies suggest that when thromboxane A_2 receptor is activated, breast cancer cells immediately contract. If the activation of this receptor is blocked, breast cancer cells cannot move and spread. We hypothesize that TP (TxA_2 receptor) regulates the motility of carcinoma cells by elaborating the reorganization of cytoskeleton during migration and that inhibition of TP activation can reduce the motility, invasion, and metastasis of breast carcinoma cells. The objective of this proposal is to define the function of TP in tumor cell motility and to validate TP as a target for anti-metastasis therapy of breast cancer with the following aims:

Aim 1. Define the role of TP activation in breast cancer cell motility.

Aim 2. Determine the isoform(s) of TP involved in cytoskeleton reorganization in motility of tumor cells.

Aim 3. Validate TP(s) as a target for treatment of breast cancer metastasis.

BODY OF REPORT

Task 1. Define the role of TP activation in breast cancer cell motility (Months 1 – 12).

Most studies in this task have been achieved in the first year of funding, and reported in the first annual report.

Task 2. Determine the isoform(s) of TP involved in cytoskeleton reorganization in motility of tumor cells (Months 13-24)

Most studies in this specific aim were completed as reported in the revised second annual report.

In the Aim 2, we attempted to generate TPbeta specific antibody but the polyclonal antibody did not work as expected, which is common in making antibody based on peptide. We later used the antibodies provided by Dr. Theresa Kinsella of Ireland to define the involvement of TP isoforms in cell motility. But the amount provided was very limited.

We have made recombinant protein covering the unique sequence of TPbeta isoform. Currently we are developing better antibody, in sufficient amount, to define TP isoform(s) involved in cell motility. Further, the antibody can be used as a tool to valuate TP expression in breast tumors to select breast cancer patients who may benefit for anti-metastasis therapy through targeting TP.

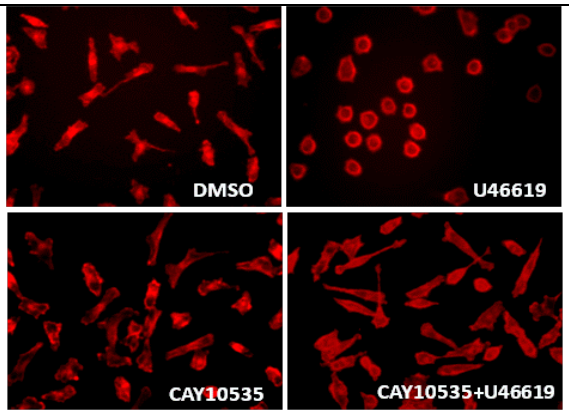

Figure 1. *Blockade of U46619-induced cell contraction by CAY10535, a select antagonist of TPβ. Cells were pretreated with CAY10535 at 100nmol/L for 15 min before the treatment with 200 nmol/L U46619 for 15 min. Cells were stained with TRITC-phallioidin.*

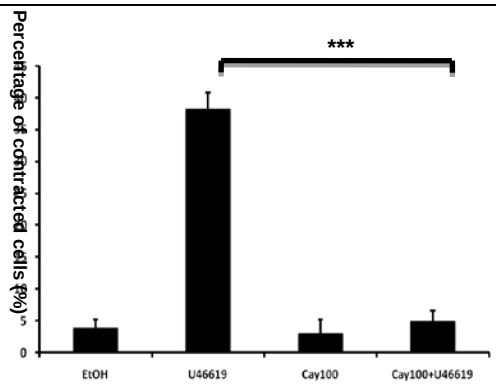

Figure 2. *Inhibition of U46619-induced cell contraction by CAY10535. Cells were pretreated with CAY10535 at 100nmol/L for 15 min before the treatment with 200 nmol/L U46619. ***, P < 0.001, when compared with its vehicle control (EtOH).*

With the availability of TPbeta selective inhibitor, CAY10535, which shows 20 fold selectivity for TPβ (IC_{50}=99nM) relative to TPα (IC_{50}=1,970nM), we determined the involvement of TPisoform involved in U46619 induced cell contraction. It was found that both low (25nM) and high (100nM) concentration of CAY10535 blocked U46619-induced cell contraction **(Figure 1**

and Figure 2). The data suggest that thromboxane A_2 mimetic U46619 induces cytoskeleton reorganization through activation of TPβ.

Further we confirmed that CAY10535 blocked the U46619 activation of RhoA using Rhotekin pulldown assay (**Figure 3**).

With our previous data suggesting the important role of RhoA in cell contraction induced by Y46619, the data suggest that TPbeta activation is required for U46619 to activate RhoA and to induce cell contraction.

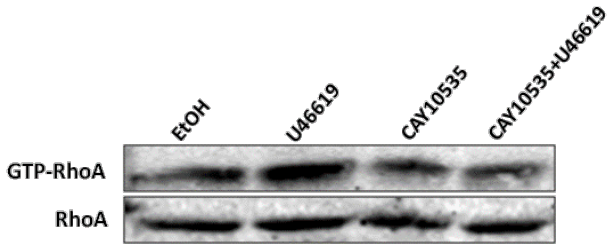

Figure 3. Inhibition of U46619 activation of RhoA by CAY10535, a select inhibitor of TPbeta. Top panel, levels of GTP-bound RhoA. Bottom panel, RhoA proteins in the supernatants after Rhotekin pull-down assay

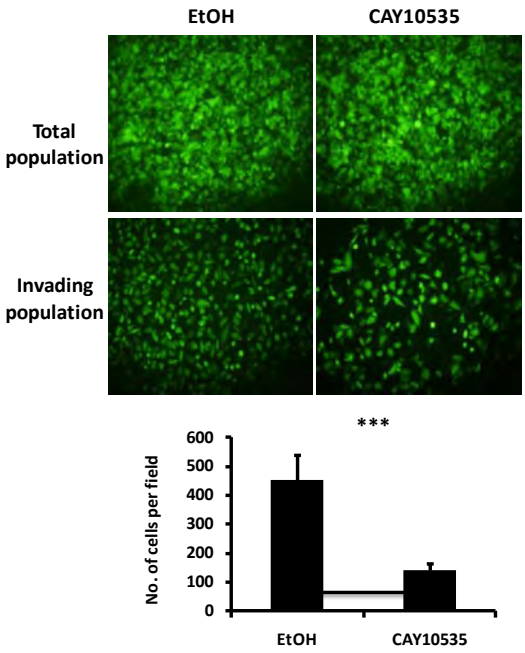

Figure 4. Inhibition of tumor cell motility by CAY10535, a select inhibitor of TPbeta. Top panel, photo micrographs depicting total population and invading populations. Bottom panel, enumeration of invaded cells. ***, $p < 0.001$.

Next we examined whether TPbeta activation is required for cell motility. As shown in **Figure 4**, CAY10535 inhibited tumor cell migration as assessed by Boyden Chamber assay.

Taken together, the data suggest TPbeta activation is required for tumor cell motility.

Task 3. Validate TP as a target for treatment of breast cancer metastasis. (Months 6 – 36). This objective will be started in the first year of funding and kept on continuous basis until the end of three year period.

In this specific aim, we have generated the MDA-MB-231 cell lines with TP stably knocked down and injected into mice to evaluate the effects of TP knockdown on tumor growth and metastasis. We found that while TP knockdown had minimal effects on the growth of primary tumors in mammary fat pads, depletion of TP significantly reduced spontaneous metastasis of MDA-MB-231 breast cancer.

To determine whether we can reduce breast cancer metastasis through pharmacological inhibitors of TP, we are trying to secure sufficient amount of SQ29548, a high affinity antagonist of TP and to treat the tumor bearing mice with the compound every other days for about 40 days (About 20 treatments). Animal experiments are being set up for this study. A no-cost extension has been requested and approved to continue the animal experiments. The results will provide us insights whether TxA2R (TP) can be a target druggable for anti-metastasis treatment of breast cancer.

KEY RESEARCH ACCOMPLISHMENT

1. Determination of the beta isoform of TP as required in cytoskeleton reorganization, small GTPase RhoA activation, and tumor cell motility.
2. Generation of TPbeta specific antibody. The polyclonal antibody did not work well in Western blot. Generation and characterization of monoclonal antibody are ongoing.
3. Animal experiments have validated TP as a target to prevent and reduce breast cancer metastasis

REPORTABLE OUTCOMES

Presentations:

Zhang Xuejing, Wang Man-Tzu, Chen Yakun, Yong Tang and Nie Daotai. Regulation of breast cancer metastasis by thromboxane A2 receptor signaling. Joint Metastasis Research Society-AACR Conference for Metastasis and the Tumor Microenvironment, Philadelphia, PA. September 12 -15, 2010. Poster presentation.

Daotai Nie. Targeting Thromboxane A2 Receptor to Block Breast Cancer Metastasis. Cold Spring Harbor Asia Conference on Translational Approaches to Cancer. Suzhou, China, May 24-27, 2011. Podium presentation.

Daotai Nie. Thromboxane A2 receptor as a target for anti-metastasis therapy of breast cancer. Depart of Defense Era of Hope Meeting. Orlando, FL. August 3 – 5, 2011. Poster presentation.

Abstracts published:

Zhang Xuejing, Wang Man-Tzu, Chen Yakun, Yong Tang and Nie Daotai. Regulation of breast cancer metastasis by thromboxane A2 receptor signaling. Proceedings of the Joint Metastasis Research Society-AACR Conference for Metastasis and the Tumor Microenvironment, 2010.

Daotai Nie, Xuejing Zhang, Man-Tzu Wang, Yong Tang, and Yakun Chen. Thromboxane A2 receptor as a target for anti-metastasis therapy of breast cancer. Proceedings of Depart of Defense Era of Hope Meeting 2011.

Articles published:

Zhang X, and D. Nie. Rho GTPases and Breast Cancer Progression. "Breast Cancer Cells / Book 4", ISBN 979-953-307-183-0. 2011. (Accepted).

Grants submitted:

Daotai Nie. Using Aspirin to Block Breast Cancer Metastasis: Mechanism of Actions and Preclinical Development. Depart of Defense Breast Cancer Research Program Idea Expansion Award.

CONCLUSIONS:

While the mRNA levels of receptor of thromboxane A_2 (TXA_2), TP, was found to correlate with a poor prognosis in breast cancer patients, it is unknown whether TP is expressed at protein levels, whether TP is functional, and whether TP regulates breast cancer progression that eventually impact on patient survival. In the past three years, we have obtained the following data suggesting the involvement of TP in breast cancer metastasis.

First, we found functional TP is expressed in breast cancer cells. Our studies identify TPbeta isoform as the receptor increasingly expressed in high grade tumors, especially in tumors with distant metastasis. TPbeta expression was found selectively limited to breast cancer cells of high malignancy as well. It is increasingly clear that TPbeta expression was stimulated as breast cancer cells become more invasive.

Second, TP activation led to cell contraction which required RhoA activation. We further determined that TPbeta is required for U46619 to induce cell contraction and RhoA activation. Identification of TPbeta in sytoskeleton reorganization and RhoA activation provides us a more specific target for possible future intervention to block breast cancer metastasis.

Third, TP activation is required for tumor cell motility. CAY10535, a select TPbeta inhibitor, inhibited tumor cell motility. The data, along with the data reported in the previous two annual reports, suggest the importance of TPbeta in tumor cell motility.

Fourth, TP is required for breast cancer metastasis. Depletion of TP reduced spontaneous metastasis in an orthotopic model, while had minimal effects on the growth of primary tumors. The results suggest TP as a potential target to reduce or block breast cancer metastasis.

The above data support the rationale of targeting TP to block breast cancer metastasis. Experiments are ongoing to determine whether inhibition of TP activation by pharmacological

inhibitors can be a valid strategy to block breast cancer metastasis. The results, once obtained, will be reported in the final report.

So what? Our studies are significant in the following ways: 1) TP, especially TPbeta, can be a promising target to develop treatment to block breast cancer metastasis. 2) Inhibition of thromboxane A2 production, either using TX synthase inhibitor or aspirin or other cyclooxygenase inhibitors may reduce breast cancer metastasis. A grant application has been submitted to Depart of Defense Breast Cancer Research Program to expand the idea of repurposing aspirin to treat breast cancer metastasis.

REFERENCES

N/A

APPENDICES

Presentations files: Not recorded

Abstracts:

Zhang Xuejing, Wang Man-Tzu, Chen Yakun, Yong Tang and Nie Daotai. Regulation of breast cancer metastasis by thromboxane A2 receptor signaling. Proceedings of the Joint Metastasis Research Society-AACR Conference for Metastasis and the Tumor Microenvironment, 2010.

Daotai Nie, Xuejing Zhang, Man-Tzu Wang, Yong Tang, and Yakun Chen. Thromboxane A2 receptor as a target for anti-metastasis therapy of breast cancer. Proceedings of Depart of Defense Era of Hope Meeting 2011.

Article:

Zhang X, and D. Nie. Rho GTPases and Breast Cancer Progression. "Breast Cancer Cells / Book 4", ISBN 979-953-307-183-0. 2011.

SUPPORTING DATA

Embedded in the reporting body

although primary s.c. tumors did not differ in size, those in SSeCKS-null mice showed increased levels of highly disorganized vasculature. Our data strongly suggest that SSeCKS suppresses parameters of metastatic neovascularization and tumor invasiveness by attenuating PKC- and Src-mediated pathways in both the tumor and stromal cell components.

A68 Regulation of breast cancer metastasis by thromboxane A_2 receptor signaling. Zhang Xuejing, Wang Man-Tzu, Chen Yakun, Tang Yong, Nie Daotai. Southern Illinois University School of Medicine and Simmons Cancer Institute, Springfield, IL.

The receptor of thromboxane A_2 (TXA_2), TP, is a member of the G-protein coupled receptor family. Previously, increased expression of TP at RNA level was found to correlate with a poor prognosis in breast cancer patients. Here we report that a strong staining of TP was noticed in infiltrating breast carcinoma using immunohistochemistry, while a much weaker staining was found in matched normal tissue. Treatment of human breast cancer cell lines MDA-MB-231 by U46619, a TP agonist, induced cell body contraction which can be blocked by high affinity TP antagonist SQ29548. Further studies found that small GTPase RhoA was activated by TP activation, and that pre-treatment of MDA-MB-231 cells with Y27632, a Rho kinase (ROCK) inhibitor, blocked U46619-induced cell contraction. The requirement of RhoA activation in U46619 induced cell contraction was further confirmed by the ability of dominant negative RhoA to block cell contraction induced by U46619. MDA-MB-231 cells with TP knockdown displayed a reduced percentage of contraction under the treatment of U46619 and TXA2 agonist I-BOP as well as attenuated invasion ability. After injection into mice via the tail vein, TP depletion reduced the ability of MDA-MB-231 cells to metastasize to the lung and the liver. TP depletion also reduced the lung colonization ability of MDA-MB-231 cells. The results indicate that TP is expressed in breast cancer and that activation of TP has profound effects on tumor cell cytoskeleton and contraction through activation of RhoA. Inhibition or depletion of TP reduces the metastatic dissemination of breast cancer cells *in vivo* by blocking the extravasation of tumor cells.

A69 Heat shock factor 1 as a multifaceted regulator of breast cancer progression and metastasis. John T. Price, Michelle M. Kouspou, Benjamin Lang, Ryan Chai, Jessica Vieusseux, Chau Nguyen. Monash University, Melbourne, Victoria, Australia.

Stress proteins, such as the heat shock proteins (Hsps), have long been associated with cancer growth and progression. Their functional importance to cancer cell biology is well documented and they have emerged as significant therapeutic targets. However, few studies have examined the role of the major transcriptional regulator of stress, heat shock factor 1 (HSF1). To determine a role for HSF1 in breast cancer (BC) progression, we screened a panel of human BC cell lines for HSF1 protein levels and activation status. Although levels of HSF1 were similar, HSF1 was highly activated in triple-negative BC cells. Expression of wild-type HSF1, constitutive active and dominant-negative mutants of HSF1 in T47D, SkBr-3 and MDA-MB-231 cells, demonstrated that HSF1 positively regulated BC cell growth, survival and migration. Moreover, activated HSF1 increased orthotopic xenograft tumour growth and enhanced experimental metastasis in the intra-cardiac inoculation model. To further investigate HSF1, we utilized MCF10A breast epithelial cells and transformed them by Ha-Ras^{v12} expression, in either the presence of exogenously expressed HSF1wt, constitutively activated HSF1 mutant or HSF1 knockdown. HSF1 knockdown negated the cell biological effects of Ha-Ras^{v12} while expression of HSF1 greatly enhanced cell growth, survival and migration, most significantly enabling cells to become highly invasive and disorganized in 3-D culture.

Due to our observed effects of HSF1 upon cell migration and the actin cytoskeleton, detected by fluorescent live cell imaging, we examined the expression levels of the small Rho GTPase, Rac1, to examine potential downstream mediators of HSF1. Consistent with HSF1 effects upon cell migration, levels of activated HSF1 positively correlated with Rac1 expression at both the RNA and protein levels. In breast cancer cells, where HSF1 had been inhibited, and associated decreases in cell migration, anchorage independent growth and reduced levels of Rac1 observed, exogenous expression of Rac1 partially rescued the migratory, growth and survival defects. Cloning and sequencing of the Rac1 promoter identified classical heat shock elements, indicating that HSF1 may be a direct regulator of Rac1 expression.

Therefore, a number of novel roles of HSF1 in breast cancer have been revealed, and Rac1 as a potential direct downstream target of HSF1 in mediating breast cancer progression. This work also provides evidence that HSF1 may constitute a novel molecular target of breast cancer impacting upon multiple facets of tumour biology.

A70 The metastasis suppressor NM23-H1 promotes genomic stability through its 3'-5' exonuclease and nucleoside diphosphate kinase activities. Stuart G. Jarrett[1], Marian Novak[1], Nathan Harris[1], Isabel Mellon[1], Sandrine Arnaud-Dabernat[2], Jean-Yves Daniel[2], David M. Kaetzel[1]. [1]College of Medicine and Markey Cancer Center, University of Kentucky, Lexington, KY, [2]Universite Victor Segalen Bordeaux 2, Bordeaux, France.

NM23-H1 is a metastasis suppressor whose reduced expression is associated with aggressive forms of melanoma, hepatoma, and carcinomas of the breast, stomach and colon. The current study has identified NM23-H1 (termed H1 isoform in human, M1 in mouse) and its 3'-5' exonuclease (3'-5' EXO) and nucleoside diphosphate kinase (NDPK) activities as novel participants in the response to UV-induced DNA damage. Kinetics of repair for total DNA polymerase-blocking lesions and nucleotide excision pathway-mediated repair of 6-4 photoproducts were significantly compromised in different cellular settings of NM23-H1-deficiency; these included a human melanoma cell line (WM793) and embryo fibroblasts (MEFs) derived from mouse strains rendered deficient in either NM23-M1 alone or both the M1 and M2 isoforms in tandem. The NDPK activity of NM23-H1 was critical for early repair of both polychromatic UVB/UVA (275–400 nm)- and UVC (254 nm)-induced DNA damage, whereas the 3'-5' exonuclease activity was required for repair of UVB/A-induced but not UVC-induced damage. Elevated rates of spontaneous and UV-induced mutation were observed in WM793 cells and NM23-deficient MEFs, and the mutational spectra reflected aberrant repair of 6-4 photoproducts and oxidative DNA damage. The 3'-5' exonuclease was the principal enzymatic activity required to reduce mutagenesis. This study has provided evidence for an essential role of NM23 isoforms in repair of UV-induced DNA damage. This novel anti-mutator function appears relevant not only to the metastasis suppressor activity of NM23-H1, but also possibly resistance to UV-induced melanomagenesis.

A71 Mdm2 is induced as cells undergo EMT and correlates with invasive late-stage breast cancer. Lindsey D. Mayo[1], Jacob Eitel[1], Karen Pollok[1], David Boothman[2], Shinako Akaki[2]. [1]Indiana University, Indianapolis, IN, [2] The University of Texas Southwestern Medical Center, Dallas, TX.

The E3 ubiquitin ligase, Mdm2, is overexpressed in 40-80% of late-stage metastatic cancers in the absence of gene amplification. Mdm2 regulates p53 stability, and Mdm2 can alter the sensitivity of cells to transforming growth factor-β1 (TGFβ1). Signal transduction pathways initiated from genotoxic stress or cell surface receptors have been shown to impact Mdm2 activity. Little is known on how TGFβ1 signaling may regulate Mdm2. In this study, we report that TGFβ1-activated Smad3/4 transcription factors specifically bind to the second promoter region of mdm2 leading to an increase Mdm2 protein levels. The elevated Mdm2 levels cause a destabilization of p53 in cancer cell lines and in murine mammary epithelial cells undergoing epithelial to mesenchymal transition (EMT). Since breast cancer cells may undergo EMT and metastasize to distal organs, we evaluated clinical samples of late-stage ductal and lobular carcinomas for activated Smad3 and Mdm2. Histological analyses demonstrate that ~65% of late-stage carcinomas are positive for activated Smad3 and Mdm2, indicating a strong correlation.

BC074897-2880

TARGETING THROMBOXANE A2 RECEPTOR TO BLOCK BREAST CANCER METASTASIS

Daotai Nie, Xuejing Zhang, Man-Tzu Wang, Yakun Chen, and Yong Tang

Southern Illinois University School of Medicine

The receptor of thromboxane A_2 (TXA_2), TP, is a seven transmembrane, G-protein-coupled receptor. Increased expression of TP at RNA level was found to correlate with a poor prognosis in breast cancer patients but it is unknown how TP expression and activities are involved in breast cancer progression. Here we report that TP is expressed in metastatic breast cancer cells at both RNA and protein levels. Treatment of breast cancer MDA-MB-231 cells by U46619, a TP agonist, induced cell body contraction. The U46619-induced contraction was blocked by SQ29548, a high-affinity TP antagonist. Further studies found that small GTPase RhoA was activated by TP activation and that pretreatment of MDA-MB-231 cells with Y27632, a Rho kinase (ROCK) inhibitor, blocked U46619-induced cell contraction. The requirement of RhoA activation in U46619 induced cell contraction was further confirmed by the ability of dominant negative RhoA to block cell contraction induced by U46619. MDA-MB-231 cells with TP knockdown displayed a reduced percentage of contraction under the treatment of U46619 and TXA_2 agonist I-BOP as well as attenuated invasion ability. When injected into mice, MDA-MB-231 cells with TP depleted did not present significant changes in primary tumor formation or growth. However, TP depletion reduced the ability of MDA-MB-231 cells to metastasize to the lung and the liver in an experimental metastasis model. We further demonstrated that TP depletion reduced the lung colonization ability of MDA-MB-231 cells by blocking active extravasation. Taken together, TP is expressed in breast cancer and that activation of TP has profound effects on tumor cell cytoskeleton and contraction through activation of RhoA. Inhibition or depletion of TP reduces the metastatic dissemination of breast cancer cells in vivo by blocking the extravasation of tumor cells.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-08-1-0540.

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Rho GTPases and Breast Cancer

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1. Introduction

The Rho GTPases is a subfamily of molecular switches that cycle between an inactive GDP-bound state and an active GTP-bound state within the Ras superfamily. In the past, members of the Rho subfamily were mainly thought to be involved in the regulation of cytoskeletal organization in response to extracellular growth factors. However, a number of studies over the past few years have revealed that the Rho GTPases play crucial roles in a wide spectrum of cellular functions related to cell adhesion to the extracellular matrix, cell morphology, cell cycle progression, malignant transformation, invasion and metastasis. Alterations of the expression levels to Rho GTPases have been detected in many types of human tumors and, in some cases, up-regulation and/or overexpression of Rho protein correlates with poor prognosis. This article reviews the evidence of aberrant Rho signaling and the cellular effects elicited by Rho GTPases signaling in human breast tumors.

2. Categorization

Rho GTPases belong to the Ras superfamily of low molecular mass (~21 kDa) proteins that are widely expressed in mammalian cells (DerMardirossian and Bokoch 2001). In mammals, the Rho family of GTPases contains 22 members which can be classified into six groups: Rho (RhoA, RhoB, RhoC), Rac (Rac1, Rac2, Rac3, RhoG), Cdc42 (Cdc42, TC10, TCL, Chp, Wrch-1), Rnd (Rnd1, Rnd2, Rnd3/RhoE), RhoBTB (RhoBTB1, RhoBTB2) and Miro (Miro-1, Miro-2) (Wennerberg and Der 2004). RhoD, Rif and RhoH/TTF have not been grouped yet. RhoA, Rac1 and Cdc42 are the best-characterized family members of Rho family GTPases. Each controls the formation of a distinct cytoskeletal element in mammalian cells. Activation of Rac induces Actin polymerization to form lamellipodia (Ridley, Paterson et al. 1992), whereas activation of CDC42 stimulates the polymerization of actin to filopodia or microspikes (Nobes and Hall 1995). In contrast, Rho regulates bundling of actin filaments into stress fibers and the formation of focal adhesion complexes (Keely, Westwick et al. 1997).

3. Regulators and effectors in Rho GTPases signaling

3.1 Regulators of the Rho GTPases

Like all members of the Ras superfamily, the activity of the Rho GTPases is tightly controlled by the ratio of their GTP/GDP-bound forms in the cell (Fig. 1)(Scheffzek and Ahmadian 2005).

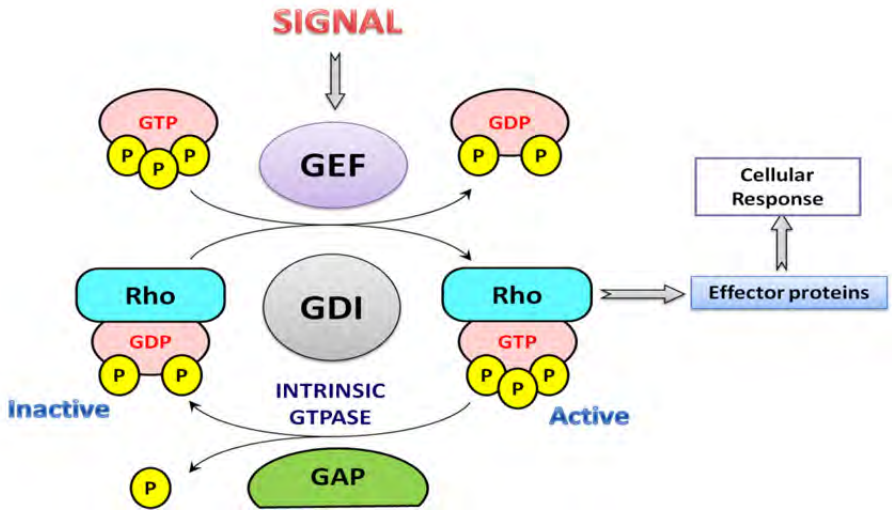

Fig. 1. Regulation of Rho family proteins.

The cycle of activation/inactivation of Rho family GTPases is under the regulation of three distinct families of proteins: GEFs, guanine nucleotide exchange factors catalyze nucleotide exchange when activated by upstream signals; GAPs, GTPase-activating proteins promote the GTP hydrolisis; GDIs, guanine nucleotide dissociation inhibitors block both nucleotide hydrolisis and exchange and participate in Rho GTPase movement between cytosol and membranes.

Rho-specific guanine nucleotide exchange factors (RhoGEFs) activate Rho proteins by facilitating the exchange of GDP for GTP. Rho GTPase activating proteins (RhoGAPs) stimulate the intrinsic rate of hydrolysis of Rho proteins, thus converting them into their inactive state. While Rho-specific guanine nucleotide dissociation inhibitors (RhoGDIs) compete with RhoGEFs for binding to GDP-bound Rho proteins, and sequester Rho in the inactive state (Olofsson 1999).

3.1.1 GEFs

GEFs for Rho GTPases belong to a rapidly growing family of proteins that share common minimal functional units, including a Db1-homolog (DH) domain followed by a pleckstrin homology (PH) domain (Cerione and Zheng 1996). The DH domain is the catalytic site required for GDP-GTP exchange, whereas the PH domain contributes to protein-protein, protein-cytoskeleton, and protein-lipid interactions that help regulate the intracellular localization of GEFs as well as their catalytic activity. Db1 oncogene product is the prototype for the DH domain, and was originally discovered because of its ability to induce focus

formation and tumorigesis when expressed in NIH-3T3 cells (Eva and Aaronson 1985). It has 29% sequence identity with the *Saccharomyces cerevisiae* cell division protein Cdc24, which is found upstream of the yeast small GTP-binding protein Cdc42 in the bud assembly pathway (Ron, Zannini et al. 1991). This was the first clue that DB1 functions as a GEF. Biochemical study has confirmed that Db1 is able to release GDP from the human homolog of Cdc42 *in vitro*. Further study suggested that the DH domain is essential and sufficient for the catalytic activity and that this domain was also necessary to induce oncogenicity (Zheng, Zangrilli et al. 1996).

After the discovery of Dbl, a number of mammalian proteins containing DH and PH domain have been studied (Cerione and Zheng 1996). Many of these have been identified as oncogenes in transfection assays. Tiam, however, was first identified as an invasion-inducing gene using proviral tagging and *in vitro* selection for invasiveness (Habets, Scholtes et al. 1994). Two other members of the DH/PH-containing protein family, Fgd1 and Vav, have been shown to be essential for normal embryonic development (Pasteris, Cadle et al. 1994; Tarakhovsky, Turner et al. 1995). Moreover, some members of the DH protein family (such as Dbl) have been shown to exhibit exchange activity *in vitro* for a broad range of Rho-like GTPases, whereas others appear to be more specific. For example, Lbc and oncoproteins Lfc and Lsc, are specific for Rho, whereas Fgd1 is specific for Cdc42 (Glaven, Whitehead et al. 1996). Although Vav was originally identified as an activator of Ras (Gulbins, Coggeshall et al. 1993), it has been demonstrated more recently to function as a GEF for members of the Rho family (Crespo, Schuebel et al. 1997; Han, Das et al. 1997).

3.1.2 GAPs

The first GAP protein specific for the Rho family GTPases was purified from cell extracts using recombinant Rho. This protein, designated p50Rho-GAP, was shown to have GAP activity toward Rho, Cdc42 and Rac *in vitro* (Hall 1990; Lancaster, Taylor-Harris et al. 1994). Since then, a growing number of proteins that present GAP activity for Rho GTPases have been identified in mammalian cells, all of which share a related GAP domain that spans 140 amino acids without significant resemblance to Ras GAP. In addition to accelerating the hydrolysis of GTP, Rho GAPs also mediate other downstream functions of Rho proteins in mammalian systems. For example, it has been reported that the p190GAP plays a role in cytoskeletal rearrangement (Chang, Gill et al. 1995).

3.1.3 GDIs

The ubiquitously expressed protein Rho GDI was the first GDI identified for the members of the Rho family. It was isolated as a cytosolic protein that preferentially associated with the GDP-bound form of RhoA and RhoB and thereby inhibited the dissociation of GDP (Fukumoto, Kaibuchi et al. 1990; Ueda, Kikuchi et al. 1990). Rho GDI was found to be active on Cdc42 and Rac as well (Abo, Pick et al. 1991; Leonard, Hart et al. 1992). Further studies demonstrated that Rho GDI also associated weakly with the GTP-bound form of Rho, Rac, and Cdc42 (Hart, Maru et al. 1992; Chuang, Xu et al. 1993), leading to an inhibition of the intrinsic and GAP-stimulated GTPase activity of the Rho GTPases. Therefore, Rho GDI appears to be a molecule capable of blocking both the GDP/GTP exchange step and the GTP hydrolytic step. It was also reported that the Rho GDIs play a crucial role in the translocation of the Rho GTPases between membranes and the cytoplasm. In resting cells, the Rho proteins are found in the cytosol as a complex with Rho GDIs, which inhibit their

GTP/GDP exchange ratio, but are released from the GDI and translocated to the membranes during the course of cell activation (Takai, Sasaki et al. 1995).

3.2 Effectors of the Rho GTPases

The Rho GTPases have been implicated in a wide varity of cellular processes, including cytoskeletal organization, cell adhesion to the substratum, cell polarity, and transcriptional activation. Several lines of evidence indicate that Rho GTPases link plasma membrane receptors to the assembly and organization of the actin cytoskeleton. Rho GTPases control individual aspects of the actin cytoskeleton through distinct effector proteins. In fact, over 60 targets of the three common Rho GTPases (Rho, Rac, Cdc42) have been found (Fig. 2).

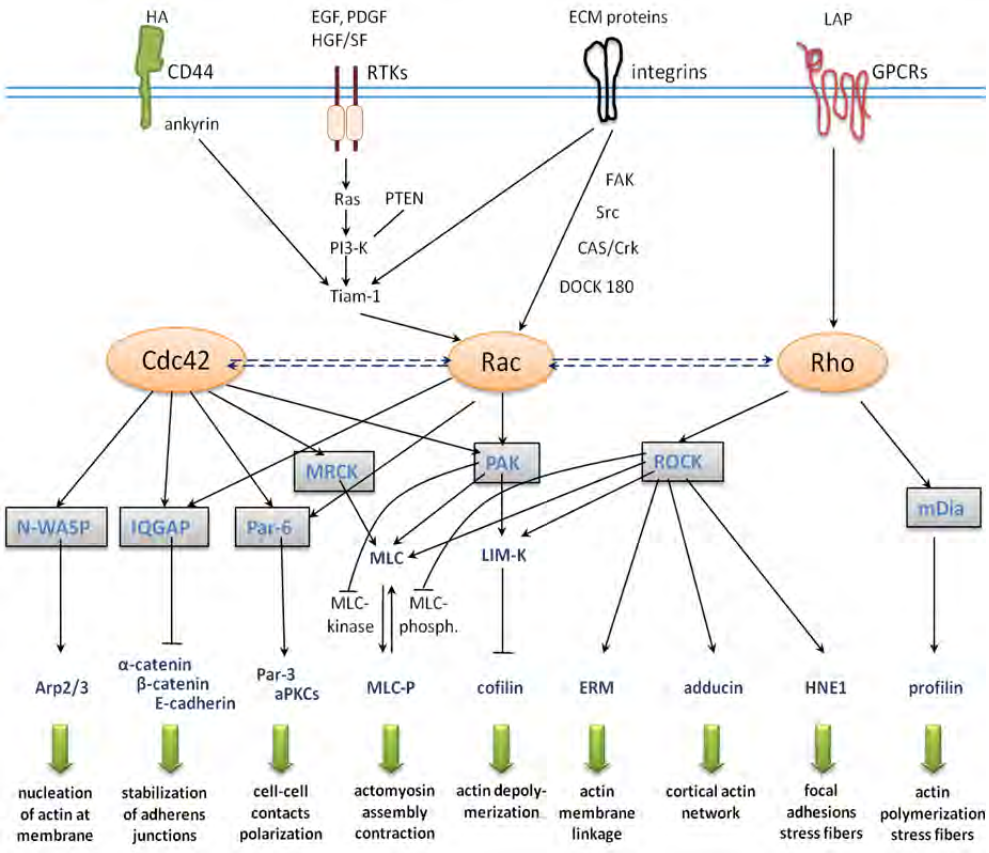

Fig. 2. Regulators and mammalian targets of the Rho family GTPases.

Transmembrane receptors activate Rho GTPases through GEFs such as Tiam-1 or adaptor proteins. Activated Rho GTPases bind to and activate protein kinases, including these of the MRCK, PAK and ROCK families. The effector proteins then interact with several proteins with distinct effects on the actin cytoskeleton and cellular morphology. See text for details.

3.2.1 Rho signaling

Rho was originally studied for its role in regulate the formation of stress fibers and focal adhesion (FA) complexes (Nobes and Hall 1995) which precursors actomyosin assembly and contractile potential, both of which are required for the cellular movement. Rho is also involved in cell-cell adhesion. In particular, inactivation of RhoA by C3 transferase disrupts the organization of actin filaments at cell-cell contact, leading to the inhibition of the proper formation of both adherens junctions (AJs) and tight junctions (TJs) (Braga, Machesky et al. 1997; Takaishi, Sasaki et al. 1997). For example, in normal mammary epithelial cells, MCF10 cells, E-cadherin cytoskeletal links in AJs was disrupted by C3 transferase. In addition, inhibition of Rho blocks the formation of new AJs in MCF10 cells (Zhong, Kinch et al. 1997). It has been suggested that the function of Rho can be either promoted or antagonized by Rac and Cdc42, depending on different variables, such as cellular context, stimulus, and extracellular matrix (ECM) (Zhang, Nie et al. ; Narumiya and Morii 1993; Nobes and Hall 1995). In Swiss 3T3 fibroblasts, the Rho GTPases have been placed in a hierarchical order where Cdc42 activates Rac, and Rac activates Rho (Nobes and Hall 1995); however, in N1E-115 neuroblastoma and Madine-Darby canine kidney (MDCK) cells, constitutively activated Rac down-regulates Rho (Leeuwen, Kain et al. 1997; Michiels and Collard 1999).

Rho is widely studied for its involvement in the acquisition of migratory, invasive, and metastatic phenotypes. Expression of a dominant negative form of RhoA led to the attenuation of membrane ruffling, lamellipodia formation and migration (O'Connor, Nguyen et al. 2000). In addition, RhoA localization to lamellipodia was blocked by inhibiting phosphodiesterase activity while enhanced by inhibiting cAMP-dependent protein kinase activity (O'Connor, Nguyen et al. 2000). Furthermore, activation of Rho either by LPA treatment or by stimulating the actomyosin system has been associated with the migratory ability of tumor cells. For example, in an experimental metastasis model, NIH3T3 fibroblasts expressing a constitutively active form of RhoA were injected into the tail vein of nude mice and formed increased number metastasis nodules in the lung (del Peso, Hernandez-Alcoceba et al. 1997). Moreover, in the absence of serum, activated RhoA is capable of promoting invasion of cultured rat MM1 hepatoma cells through a mesothelial cell monolayer (Yoshioka, Matsumura et al. 1998). Although these are not oncogenes by themselves, RhoA and RhoC are frequently found to be overexpressed in clinical cancers (Sahai and Marshall 2002), and RhoC has been repeatedly associated with metastasis. For example, the expression of RhoA, RhoB and RhoC in 33 pancreatic ductal adnocarcinoma cases were examined in a study (Suwa, Ohshio et al. 1998), it was found that the expression level of RhoC was higher in tumors than in non-malignant tissues, higher in metastatic lesions than in primary tumors, and correlated with perineural invasion and lymph node metastasis as well as poorer prognosis. Although early studies showed that RhoB has a positive role in cell growth, more recent studies suggested that RhoB is down-regulated in human tumors, and its expression inversely correlates with tumor aggressiveness. For example, RhoB protein is found expressed in normal lung tissue and is lost progressively throughout lung cancer progression (Mazieres, Antonia et al. 2004). In line with this, higher expression of RhoB is associated with favorable prognosis in bladder cancer (Kamai, Tsujii et al. 2003). It has been suggested that RhoB can act as a tumor suppressor, since it is activated in response to several stress stimuli, such as DNA damage and hypoxia, inhibits tumor growth, cell migration, and invasion, and has proapoptotic functions in cells (Huang and Prendergast 2006).

3.2.2 Effectors of Rho

There are two major effectors that are downstream of Rho: Rho associated coiled-coil forming kinase (ROCK/Rho kinase/ROK) (Leung, Manser et al. 1995; Ishizaki, Maekawa et al. 1996) and mammalian homolog of Drosophila diaphanous (mDia) (Watanabe, Madaule et al. 1997; Wasserman 1998). While mDia is a formin molecule that can catalyze actin nucleation, polymerization,and produce long, straight actin filaments (Goode and Eck 2007), ROCK is a serine/threonine kinase that phosphorylates a number of substrates (Riento and Ridley 2003). The actions of ROCK and mDia on actin and myosin are believed to work together to induce actomyosin bundles in cells. Expression of an active form of mDia induces stress fibers in cultured cells, and treatment of these cells with a specific ROCK inhibitor, Y-27632 (Narumiya, Ishizaki et al. 2000), causes dissolution of the bundles, leaving the cells with diffusely distributed actin filaments (Watanabe, Kato et al. 1999). It has also been reported that ROCK and mDia are required in contractile ring formations (Kosako, Yoshida et al. 2000; Watanabe, Ando et al. 2008).

At least six substrates of ROCK are known to play roles in actin cytoskeletal reorganization, including myosin light chain (MLC), myosin-binding subunit of MLC phosphatase, LIM-kinase, adducin, ezrin/radixin/moesin (ERM) family of proteins, and Na^+/H^+ exchange protein (NHE1). Among the six substrates, MLC-phosphatase, MLC, and LIM-kinase, are the three best studied ROCK effectors and have been found to play important roles in driving ROCK's physiological function on the actin cytoskeleton. ROCK inactivates myosin-binding subunit of MLC-phosphatase by phosphorylation (Kimura, Ito et al. 1996; Uehata, Ishizaki et al. 1997). ROCK is also able to phosphorylate myosin light chain directly (Maekawa, Ishizaki et al. 1999). These two actions of ROCK increase the myosin light chain phosphorylation, stimulate cross-linking of actin by myosin and enhance actomyosin contractility. ROCK also phosphorylates and activates LIM-kinase, which in turn phosphorylates and inactivates actin-depolymerizing and severing factor, cofilin (Amano, Ito et al. 1996). The later action of ROCK results in stabilization of existing actin filaments and increase in their content.

The ROCK effectors adducin and the ERM family of proteins regulate actin cytoskeleton in a more direct way. ROCK has been shown to phosphorylate adducin (Kimura, Fukata et al. 1998; Fukata, Oshiro et al. 1999), which, together with spectrin, is an important component of the cortical actin network underlying the plasma membrane (Gardner and Bennett 1987). ROCK-phosphorylated adducin interacts with filamentous-actin (F-actin), and its localization suggests a role in regulating cellular migration. In HGF/SF-stimulated MDCK cells, phosphoadducin localizes to membrane ruffles, and ROCK-phosphorylated adducin localizes to the leading edge of migrating NRK49F fibroblasts in wound healing assays (Fukata, Oshiro et al. 1999); while the introduction of nonphosphorylatable adducin into MDCK and NRK49F cells inhibited membrane ruffling and migration, as did a dominant negative ROCK mutant (Fukata, Oshiro et al. 1999). ROCK can also phosphorylate the ERM proteins that are important for linking actin filaments to the plasma membrane (Matsui, Maeda et al. 1998). Interestingly, it has been demonstrated that the TSC1 tumor suppressor hamartin regulates cell adhesion to cell substrates through the ERM family of actin-binding proteins and RhoA (Lamb, Roy et al. 2000). Finally, NHE1 is well known as a ubiquitous Na^+/H^+ exchange protein that enables stress fiber formation (Tominaga, Ishizaki et al. 1998).

3.2.3 Rac and Cdc42 signaling

In classical Swiss 3T3 fibroblast model, activation of Cdc42 leads to filopodia formation, Rac results in lamellipodia formation and membrane ruffling, and Rho results in stress fibers formation (Nobes and Hall 1995). The cytoskeletal rearrangements caused by Rho GTPases activation play a key role in cell motility. In addition to their effects on the actin cytoskeleton and motility, Rac and Cdc42 also play a role in cell-cell adhesion in epithelial cells. Expression of a constitutively active form of Rac in MDCK cells or keratinocytes leads to an increase in E-cadherin complex members and F-actin at cell-cell contacts, while a dominant negative mutant was found to disrupt cell-cell adhesions (Braga, Machesky et al. 1997; Takaishi, Sasaki et al. 1997; Jou and Nelson 1998). A number of studies have suggested that Cdc42 plays an important role in establishing the initial polarization of epithelial cells, which is required for the proper formation of cell-cell adhesions. For example, transfection of a dominant negative form of Cdc42 in MDCK cells results in the selective depolarization of basolateral membrane proteins due to inhibition of membrane transport (Kroschewski, Hall et al. 1999). Expression of a constitutively active form of Cdc42 in MDCK cells increased AJs and blocked cellular migration induced by HGF/SF (Kodama, Takaishi et al. 1999).

Given the importance of Rac and Cdc42 in the regulation of cell cytoskeletal, adhesion and motility, it has been widely considered that they play important roles in cellular processes related to invasion and metastasis. The first evidence of Rac's role in invasion was obtained when Rac-specific GEF T-lymphoma invasion and metastasis (Tiam-1) was identified in a retroviral insertional mutagenesis screen. Virus-infected T-lymphoma cells were repeatedly selected for *in vitro* invasion through a layer of fibroblasts and the proviral insertions in invasive clones were used to identify the Tiam-1 gene (Habets, Scholtes et al. 1994). Subsequently, Rac, and later Cdc42, were shown to also confer an invasive potential to these T-lymphoma cells (Michiels, Habets et al. 1995; Stam, Michiels et al. 1998). More evidence for Rac and Cdc42's involvement in invasion and metastasis has been provided since then. Expression of the laminin-receptor α6β4 integrin in the melanoma cell line MDA-MB-435 promotes invasiveness in a Rac and PI3-kinase-dependent manner (Shaw, Rabinovitz et al. 1997). In addition, constitutively active forms of Rac and Cdc42 in breast carcinoma cell line T47D promote invasion through a collagen matrix. However, this invasion can be blocked by PI3-Kinase inhibitors, indicating that PI3-kinase acts downstream of Rac and Cdc42 (Keely, Westwick et al. 1997).

3.2.4 Effectors of Rac and Cdc42

A number of Rac and Cdc42 effectors have been identified. Some of these have been found to specifically mediate cell motility, whereas others play a more prominent role in mediating cell adhesion. It is well established that WASP and MRCKs are Cdc42 specific effectors that regulate actin organization and filopodia formation which promotes a more motile phenotype (Aspenstrom, Lindberg et al. 1996; Miki, Miura et al. 1996). In addition, members of the p21-activated kinase family (PAK), downstream of Rac and Cdc42, play important roles in cytoskeletal-mediated changes that affect motility (Manser, Leung et al. 1994). The scalffold proteins IQGAP and Par-6, both of which can be activated by Cdc42 and rac, promote cell polarization and contribute to cell-cell adhesion.

The scaffold protein N-WASP binds to Arp2/3 complexes that are crucial for the assembly of within filopodia (Kolluri, Tolias et al. 1996). It has been shown that both N-WASP and Arp2/3 complexes are required for Cdc42 to trigger actin filament assembly (Welch, DePace

et al. 1997; Miki, Sasaki et al. 1998). Therefore, N-WASP may promote cellular motility through proper filopodia formation. MRCKs α and β are Cdc42 specific effectors that can phosphorylate MLC via a ROCK-like kinase domain (Leung, Chen et al. 1998). It is well accepted that phosphorylation of MLC is required for actomyosin complex assembly and contraction. Overexpression of MRCKα and Cdc42 synergizes to promote filopodia formation, while a MRCKα kinase-deficient mutant inhibits the formation of Cdc42-induced filopodia (Leung, Chen et al. 1998). Therefore, MRCDs are believed to play important roles in cytoskeletal organization and contraction, and contribute to migration. PAK, a protein kinase downstream of Rac and Cdc42, plays a crucial role in actin dynamics and adhesion (Manser, Leung et al. 1994). PAK has been demonstrated to phosphorylate and inactivate MLCK, subsequently causing a decrease in MLC phosphorylation (Sanders, Matsumura et al. 1999). Thus, inactivation of MLCK leads to stress fiber and focal adhesion disassembly. Moreover, PAK controls the actin cytoskeletion through the phosphorylation and subsequent activation of LIM-kinase. Phosphor-LIM-kinsae can further phosphorylate and inactivate the actin-depolymerizing protein cofilin, thus inhibiting actin depolymerization when Rac is activated and causing extreme membrane ruffling (Arber, Barbayannis et al. 1998; Yang, Higuchi et al. 1998). The IQGAP1 and IQGAP2 scaffolding effectors of Cdc42 and Rac regulate cell-cell adhesion through actin polymerization and sequestration of β-catenin (Kuroda, Fukata et al. 1996; Erickson, Cerione et al. 1997). *In vitro*, IQGAP oligomerizes and cross-links F-catin it has also been found to complex with Cdc42 and F-actin *in vivo* (Fukata, Kuroda et al. 1997). In addition, one study has shown that the IQGAP protein also competes with α-catenin for binding to β-catenin, thus preventing E-cadherin/α-catenin/β-catenin complex from attaching to the actin cytoskeleton, and thereby disrupting cell-cell contacts (Erickson, Cerione et al. 1997). Another scaffolding protein, Par-6, was identified using activated Cdc42 and TC10 mutants as baits in yeast two-hybrid screens (Joberty, Petersen et al. 2000; Qiu, Abo et al. 2000). It is known that Par-6 binds to a second scaffolding protein, Par-3, and both Par-6 and Par-3 bind independently to atypical protein kinase C (aPKC) isioforms (Lin, Edwards et al. 2000). In addition, endogenous Par-3 localizes to TJs in MDCK cells, overexpression of Par-6 or the N-terminal portion of Par-3 (the Par-6-interaction responsible region) disrupts TJ formation (Joberty, Petersen et al. 2000).

4. Expression of Rho GTPases in breast tumors

Aberrant Rho signalling resulting from alterations in Rho GTPase protein level, changes in activation status, and abnormal quantity of effector proteins are found in a large variety of human tumors. of GTPases: the Rho family (RhoA, RhoB and RhoC), the Rac family (Rac1, Rac2 and Rac3) and the Cdc42 family, in order to avoid repetitions.

4.1 Rho GTPases in breast tumors

Overexpression of RhoC has been found in inflammatory breast cancer (IBC), an aggressive form of breast cancer that is highly infiltrative and metastatic with poor prognosis for the patients, using in situ hybridization (van Golen, Davies et al. 1999). Compared to normal untransformed parental cells, RhoC-transformed cells produce and secrete high levels of proangiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), interleukin-6 (IL-6), and interleukin-8 (IL-8). when compared to normal untransformed parental cells (van Golen, Wu et al. 2000). In addition, microarray

analysis has shown that MCF10A breast cells stably transfected with wild type RhoC or a constitutively active mutant of RhoC overexpress genes associated with invasion and metastasis (Wu, Wu et al. 2004). Other RhoGTPases are also involved in breast tumors. RhoA is found overexpressed in breast tumor tissues but not in the normal tissue (Fritz, Brachetti et al. 2002). The expression of dominant negative RhoA in rat mammary adenocarcinoma cells affects tumor cell growth *in vivo* and reduces intravasation into the peripheral blood, resulting in decrease in lung colonization ability (Fritz, Just et al. 1999). Other studies have indirectly shown an important role of RhoA in breast carcinogenesis. For example, highly metastatic MDA-MB-231 cells that were treated with HMG-CoA reductase inhibitor, namely cerivastain, showed reduced proliferation and invasion through Matrigel, in a RhoA- but not Ras-dependent manner (Denoyelle, Vasse et al. 2001). However, poorly metastatic breast cancer cells such as MCF-7 are less sensitive to cerivastain treatment, indicating that RhoA might be more significantly overexpressed in late stages of breast cancer as with other tumors.

Rho proteins are also important players in breast tumor progression and metastasis exerted by the CD44 hyaluronan receptor (Bourguignon 2001). CD44 is expressed in human breast tumors and promotes cell growth and metastasis in tumor cells. Studies have found that RhoA and CD44 directly interact with each other *in vivo* in highly metastatic human breast cancer cell lines. Accordingly, inhibition of Rho signaling results in the abrogation of the metastatic phenotype elicited by CD44 (Bourguignon, Zhu et al. 1999). RhoA has also been found to be involved in insulin signaling via Shc in human breast cancer (Finlayson, Chappell et al. 2003). Overexpression of insulin receptors correlates with development, progression and outcome of breast cancer, and insulin signaling involves hyperphosphorylation of Shc. Hence, Shc leads to the activation of geranyl transferases, which results in an increased amount of prenylated RhoA in the tumor tissue compared with normal mammary tissue (Finlayson, Chappell et al. 2003). Furthermore, RhoA has been reported to increase the metastatic potential of tumor cells via its ability to promote tumor angiogenesis through the downregulation of thrombospodin-1 (Tsp-1) (Watnick, Cheng et al. 2003). Rho pathway is part of the downstream signaling cascade that is activated by PI3K and leads to ROCK stimulation, Myc phosphorylation and Tsp-1 repression.

4.2 Rac GTPases in breast tumors

The involvement of Rac GTPases in breast cancer was first reported in rodents (Bouzahzah, Albanese et al. 2001). Expression of a dominant negative Rac1 mutant indicated that Rac1 affects tumor cell growth and metastasis *in vivo*. Deregulation of Rac3, closely related to Rac1, has also been detected in breast cancer (Mira, Benard et al. 2000). Rac3 maps to chromosome band 17q25.3, a region known to contain candidate tumor suppressor genes both in breast and ovarian cancers (Morris, Haataja et al. 2000). Highly proliferative breast cancer cells, T47D and MCF-7, but not normal breast cell lines, contain constitutively active Rac3 in a Ras-independent manner (Morris, Haataja et al. 2000). It has also been shown that expression of a dominant negative mutant Rac3 (N17) leads to inhibition of S-phase entry and cellular proliferation in breast tumor cells, which indicate that Rac3 may promote cell growth (Leung, Nagy et al. 2003). Further, the Rac-PAK signaling pathway is essential for receptor tyrosine kinase ErbB2-mediated transformation of human breast epithelial cancer cells (Mazieres, Antonia et al. 2004). Activation of Rac-PAK1 pathway by ErbB2 homodimers can induce growth factor-independent proliferation and promote disruptions to the three-dimensional (3D) mammary acinar-like structures, via activation of the Erk and

Akt pathways (Mazieres, Antonia et al. 2004). Moreover, Rac1 enhances estrogen receptor α (ERα) transcriptional activity, resulting in increased proliferation in breast cancer cells (Rosenblatt, Garcia et al. ; Folkman 1972).

4.3 Cdc42 family in breast tumors

Cdc42 is overexpressed in some breast cancers and there is accumulating evidence that activated Cdc42 contributes to the accumulation of ErbB1 in cells through the regulation of c-Cbl function (Abraham, Kuriakose et al. 2001; Marionnet, Lalou et al. 2003). The view that Cdc42 is involved in human breast carcinogenesis is supported by a rodents model of breast carcinoma where the expression of a dominant negative mutant of Cdc42 reduced the number of focal contacts, inhibited colony formation in soft agar and affected cell growth *in vivo* (Fritz, Just et al. 1999). The dominant negative Cdc42 also reduced intravasation of tumor cells into peripheral blood and ability to form lung metastasis. In addition, through the activation of Cdc42, transforming growth factor α (TGF-α) mediates the invasion of MDA-MB-231 cells into 3-D collagen matrices by initiating the formation of protrusions into collagen. (Kamai, Tsujii et al. 2003; Fisher, Sacharidou et al. 2009). Further, another study has shown that membrane-type-1 matrix metalloproteinase (MT1-MMP) and Cdc42 are fundamental components of a co-associated invasion-signaling complex that controls directed single-cell invasion of 3D collagen matrices (Fisher, Sacharidou et al. 2009).

5. Multiple functions mediated by Rho GTPases in breast cancer

Rho GTPases mediate housekeeping aspects of cell biology including cell growth, cell polarity, cell adhesion, membrane trafficking and motility. They function as signaling switches that regulate lipid metabolism, microtubules- and actin-based structures, epithelial cell-junctions, cell cycle and apoptosis regulatory proteins, and transcription factors.

5.1 Rho GTPases and cytoskeleton organization

Eukaryotic cellular morphology and attachment to the substratum in response to extracellular signals are largely dependent on rearrangements of the actin cytoskeleton. Cell motility, cytokinesis and phagocytosis all rely on coordinated regulation of the actin cytoskeleton (Small 1994; Zigmond 1996). Filamentous actin can be organized into several discrete structures: (a) filopodia, finger-like protrusions that contain a tight bundle of long actin filaments in the direction of the protrusion. These are found primarily in motile cells and neuronal growth cones. (b) lamellipodia, thin protrusive actin sheets that dominate the edges of cultured fibroblasts and many other motile cells. Membrane ruffles observed at the leading edge of the cell result from lamellipodia that lift up off the substrate and fold backward. (c) actin stress fibers, bundles of actin filaments that traverse the cell and are linked to the ECM through focal adhesion (Van Aelst and D'Souza-Schorey 1997). The actin polymerization is tightly regulated by Rho GTPases.

Rho activation in fibroblasts is known to stimulate the assembly of contractile actin/myosin filaments, the formation of stress fibers, and the clustering of integrins involved in the formation of focal adhesion complexes. Activation of Rac facilitates actin polymerization at the cell periphery to generate protrusive actin-rich lamellipodia and membrane ruffling. And activation of Cdc42 results in actin polymerization to form peripheral actin microspikes and filopidia. As described previously, a number of proteins have been identified as targets

of Rho, Rac and Cdc42 (Fig. 3). Most of them are involved in Rho GTPases mediated cytoskeletal rearrangements (Tang, Olufemi et al. 2008).

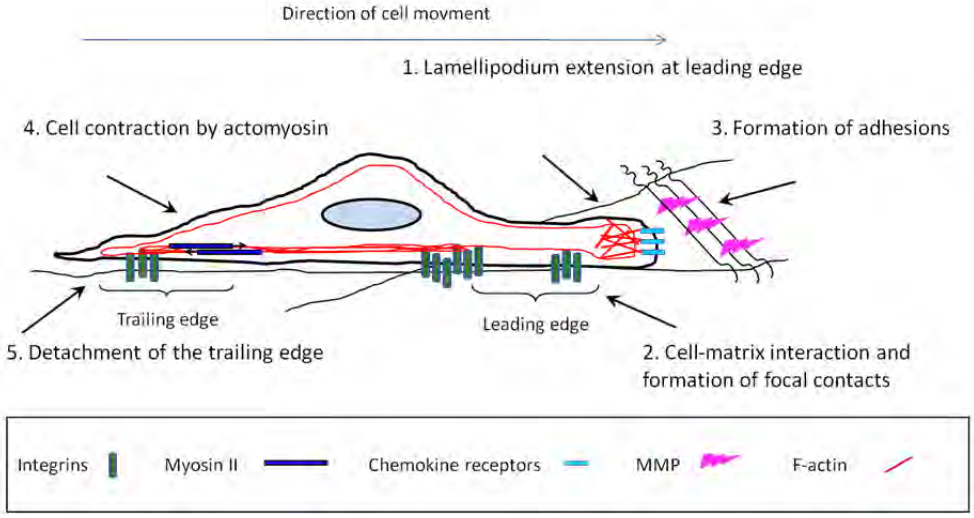

Fig. 3. A model of the cellular migratory process. See text for detailed explanation of motility phases.

5.2 Rho GTPases in cell migration

Cell migration is a multistep process involving polarization, sequential cell protrusion and adhesion formation in the direction of migration, cells body contraction, and tail detachment (Pinner and Sahai 2008). During the migration process, cells move with extending protrusions at the front and a retracting tail at the rear, both regulated by members of the Rho GTPases family (Ridley, Schwartz et al. 2003). The idea that Rho family GTPases could regulate cell migration derives from observations that they mediate the formation of specific actin containing structures. In addition, Rho proteins regulate several other processes that are relevant to cell migration, including cell-substrate adhesion, cell-cell adhesion, protein secretion, vesicle trafficking, and transcription.

5.2.1 Cell polarization and lamellipodium extension at the leading edge

An asymmetrical organization of intracellular activities is required for a cell to move, that means the molecular processes at the leading and trailing edges of a moving cell must be different. Establishing and maintaining cell polarity in response to extracellular stimuli appear to be mediated by Rho family GTPases.

Cdc42 is well accepted as a master regulator of cell polarity in eukaryotic organisms ranging from yeast to human.Cdc42 was first studied in a budding yeast model for its involvement in cell polarity. During the cell cycle, yeast cells adopt alternative states of growth to non-focused isotropic growth. In the absence of Cdc42, *Saccharomyces cerevisiae* fail to establish focused apical growth and, cells expand isotropically (Pruyne and Bretscher 2000). Cdc42 regulates cell polarity by deciding the location of lamellipodia formation (Srinivasan, Wang et al. 2003). In addition, Cdc42 directs the localization of the microtubule-organizing center

(MTOC) and Golgi apparatus to the front of the nucleus, oriented toward the direction of movement. MTOC orientation at the leading edge then facilitates the delivery of Golgi derived vesicles to the leading edge and microtubule growth into the lamellipodium (Rodriguez, Schaefer et al. 2003). It has been further studied that Cdc42 exerts its effect on MTOC through its downstream effector, PAK1 (Li, Hannigan et al. 2003).

5.2.2 Protrusion formation

Inherent polarity drives the formation of membrane protrusions, and the organization of filaments depends on the type of protrusion. Actin filaments form a branching dendritic network in lamellipodia, but form long parallel bundles in filopodia (Pollard, Blanchoin et al. 2000). The dendritic organization of lamelipodia that provides a tight brush-like structure, formed via the actin-nucleating activity of the actin-related proteins 2/3 (Arp2/3) protein complex (Urban, Jacob et al.). Rac stimulates new actin polymerization by acting on Arp2/3 complexes, which binds to pre-existing filaments (Campellone and Welch). Activation of Arp2/3 complexes by Rac is carried out through its target IRSp53. Upon activation, IRSp53 interacts with WAVE through its SH3 domain, it then binds to and activates Arp2/3 complexes (Chesarone and Goode 2009). It has also been reported that IRSp53 binds to Cdc42 through a separate domain (Miki, Yamaguchi et al. 2000). So, IRSp53 can serve as a direct link between Cdc42 and Rac, which explains how Cdc42 induces Rac involvement in lamellipodium formation. Furthermore, IRSp53 can bind to a Rho target, Dial, which might underlie the capability of Rho to facilitate lamellipodium extension (Cox and Huttenlocher 1998; Fujiwara, Mammoto et al. 2000).

5.2.3 Cell-substrate adhesions

Newly formed focal adhesion complexes are localized in the lamellipodia of most migrating cells. Once the lamellipodium attach to the ECM, integrins come into contact with ECM ligands and cluster in the cell membrane where they interact with FAK, α-actin, and talin (Cox and Huttenlocher 1998). All these proteins can bind to adaptor proteins through Src-homologous domain 2 and 3 (SH2, SH3) as well as proline rich domains to more actin binding proteins (vinculin, paxillin and α-actin) and regulatory molecules PI3K to focal complexes (Zamir and Geiger 2001). Rac is required for focal complex assembly, and Rac itself can be activated by cell-substrate ECM adhesion (Rottner, Hall et al. 1999). It is suggested that the adhesion assemblies in migrating cells begin with small-scale clustering and the speed of the cell migration is dependent on ECM composition, which determines the relative activated levels of Rho, Rac and Cdc42 (Price, Leng et al. 1998). Therefore, interactions between ECM and integrins at the leading edge of cells play an important role in maintaining the level of active Rac. This indicates the existence of a positive feedback loop that allows continuous crosstalk between integrins and Rac, and allows cells to respond to changing ECM composition.

5.2.4 Cell body contraction by actomyosin complexes

Cell body contraction is driven by actomyosin contractility and the force transmitted to sites of adhesion derives from myosin II. Myosin II, which is predominantly induced by Rho and its downstream effector ROCK, controls stress fiber assembly and contraction. Rho acts via ROCKs to affect MLC phosphorylation by inhibiting MLC phosphatase or the MLC phosphorylation. MLC phosphorylation is also regulated by MLCK, which is controlled by both intracellular calcium concentration and ERK MAPKs (Fukata, Amano et al. 2001).

ROCKs and MLCK have been suggested to act in concert to regulate different aspects of cell contractility, since ROCK appears to be required for MLC phosphorylation which are associated with actin filaments in the cell body, and MLCK is required at the cell periphery (Totsukawa, Yamakita et al. 2000).

5.2.5 Adhesion disassembly and tail detachment

Tail detachment occurs when cell-substrate linkages are preferentially disrupted at the rear of a migrating cell, while the leading edge remains attached to the ECM and continues to elongate (Palecek, Huttenlocher et al. 1998). Mechanisms underlying the focal complex disassembly and tail detachment depend on the type of cell and strength of adhesion to the extracellular matrix at the trailing edge (Wear, Schafer et al. 2000). In slow moving cells, tail detachment depend on the action of a calcium-dependent, non-lysosomal cysteine protease calpain that cleaves focal complex components like talin and cytoplasmic tail of β1 and β3 integrins along the trailing edge (Potter, Tirnauer et al. 1998). Strong tension forces exerted across the cells at the rear adhesions is required to break the physical link between integrin and the actin cytoskeleton. Rho and myosin II are involved in this event. Furthermore, Rho plays important roles in reducing adhesion and promoting tail detachment in fibroblasts, which have relatively large focal adhesion complexes (Cox and Huttenlocher 1998).

5.3 Rho GTPases and transcriptional activation

A number of studies have suggested that Rho family GTPases are involved in the regulation of nuclear signaling. Rac and Cdc42, but not Rho, have been demonstrated to regulate the activation of JNK and reactivate kinase p38RK in certain cell types (Seger and Krebs 1995). Expression of constitutively active forms of Rac and Cdc42 in HeLa, NIH-3T3, and Cos cells stimulates JNK and p38 activity (Coso, Chiariello et al. 1995). Furthermore, these same effects were observed with oncogenic GEFs for these Rho proteins (Minden, Lin et al. 1995). However in human kidney 293 T cells, Cdc42 and the Rho protein, but not Rac, induces the activation of JNK (Teramoto, Crespo et al. 1996). Upon activation, JNKs and p38 translocate to the nucleus where they phosphorylate transcription factors, including c-Jun, ATF2, and Elk (Derijard, Hibi et al. 1994; Gille, Strahl et al. 1995). Further, Rac has been shown to activate PEA3, a member of the Ets family, in a JNK-dependent manner (O'Hagan, Tozer et al. 1996). Activated p38 phosphorylates ATF2, Elk, Max, and the cAMP response element binding protein.

PAKs are the farthest known upstream kinases that connect Rho GTPases to JNK and p38 through GTP-dependent bindings to Rac and Cdc42 *in vitro* and are activated after binding to activated Rac and Cdc42. (Manser, Chong et al. 1995). In addition, certain constitutively active forms of PAK can activate JNK and p38 (Zhang, Han et al. 1995). Further, a mutant effector of Rac that cannot bind to PAK remains a potent JNK activator (Westwick, Lambert et al. 1997). These observations suggest that other kinases, in addition to PAK, participate in the signalling from Rho GTPases to JNK. Supporting this, MLK3 and MEKK4 are found to be regulated by Cdc42 and Rac, and selectively activate the JNK pathway (Gerwins, Blank et al. 1997). It has also been reported that Cdc42/Rac can bind to MLK3 both *in vitro* and *in vivo* and that the coexpression of activated Cdc42/Rac mutants elevates the enzymatic activity of MLK3 in Cos-7 cells (Teramoto, Coso et al. 1996; Gerwins, Blank et al. 1997). In addition, Rho, Rac and Cdc42 stimulate the activation of the serum responsive factor (SRF) (Hill, Wynne et al. 1995). SRF forms a complex with TCF/Elk proteins to stimulate transcription

with serum response elements (SREs) at their promoter enhancer regions, for example the Fos promoter (Treisman 1990).

5.4 Rho GTPases and cell growth control

Several lines of evidence have suggested that Rho family members play important roles in several aspects of cell growth. The Rho proteins have been shown to increase expression of cyclin D1, a cell cycle regulator that controls the transition from G_1 phase to S phase, in Swiss 3T3 fibroblasts (Yamamoto, Marui et al. 1993; Olson, Ashworth et al. 1995) and in mammary epithelial cells (Liberto, Cobrinik et al. 2002). Overexpression of RhoE inhibits cell cycle progression by inhibiting translation of cyclin D1 mRNA (Villalonga, Guasch et al. 2004). In fibroblasts, RhoA is involved in ERK activation and subsequent cyclin D1 expression (Roovers and Assoian 2003). RhoA also downregulates cdk inhibitors p21 and p27 during the G1 phase of the cell cycle (Weber, Hu et al. 1997). Rac 1 is capable of regulating the cell cyle through the activation of a number of distinct intra-cellular pathways, including the NFκB pathway. In contrast to other Rho proteins, Rac1 can directly activate cyclin D1 expression (Page, Li et al. 1999).

Furthermore, Rho, Rac, and Cdc42 have been demonstrated to possess transforming and oncogenic potential in some cell lines. For example, cells with constitutively active forms of Rac and Rho display enhanced anchorage independent growth ability, and initiate tumor formation when inoculated into nude mice (Khosravi-Far, Solski et al. 1995). The observation that Tiam, a Rac GEF, can transform NIH-3T3 cells suggests a role for Rac in transformation (van Leeuwen, van der Kammen et al. 1995). While expression of constitutively activated Rac is sufficient to cause malignant transformation of rodent fibroblasts (Qiu, Chen et al. 1995), this is not the case with Rho (Qiu, Chen et al. 1995), suggesting that the growth-promoting effects of the Rho GTPases are specific to cell type. Evidence of Cdc42's role in cell growth has been provided in fibroblasts. The constitutively active mutant of Cdc42 stimulates anchorage independent growth and proliferation in nude mice (Qiu, Abo et al. 1997). Using a Cdc42 mutant, Cdc42(F28L), that can undergo GTP-GDP exchange in the absence of GEF, one study demonstrated that cells stably tranfected with Cdc42(F28L) exhibited not only anchorage-independent growth but also lower dependence on serum for growth (Lin, Bagrodia et al. 1997). A role for Cdc42 in Ras transformation has also been established in Rat 1 fibroblasts. Coexpression of a dominant negative form of Cdc42, Cdc42N17, with oncogenic Ras results in inhibition of RasV12-induced focus formation and anchorage-independent growth, and reversed the change in morphology in RasV12-transformed cells (Qiu, Abo et al. 1997).

5.5 Rho GTPases and angiogenesis

Beside their roles in multiple processes of cellular control, tumor growth, progression and metastasis, the Rho proteins have also been shown to be involved in angiogenesis, a process Where new blood vessels arise from existing mature vessels. This process is controlled by a number of pro-angiogenic and anti-angiogenic factors at different stages (Folkman 1972). The major pro-angiogenic factors are comprised of vascular endothelial growth factor (VEGF), fibroblast growth factors (FGF), platelet derived growth factor-β (PDGFβ), angiopoietins 1 and 2 (Ang-1 and 2), tumor necrosis factor (TNF), interleukin 6 and 8 (IL-6 and 8), and epidermal growth factor (EGF). The main anti-angiogenic foctors include the thrombospondins (TSPs), angiostatin, and endostain (Merajver and Usmani 2005). The Rho

proteins are believed to be capable of altering the expression and activity of pro-angiogenic and anti-angiogenic factors during angiogenesis.

5.5.1 Regulation of VEGF and hypoxia inducible factor-1 (HIF1)

It has been reported that hypoxia increases the expression and activity of Cdc42, Rac1 and RhoA in renal cell carcinoma cell lines and a human microvascular endothelial cell line (Turcotte, Desrosiers et al. 2003). This study demonstrated that reactive oxygen species (ROS) are responsible for the upregulation of Rho proteins and that RhoA is required for the accumulation of HIF-1α (Turcotte, Desrosiers et al. 2003), a transcription factor induced by hypoxia that plays important roles in the process of angiogenesis by inducing the transcription of crucial mediators, including VEGF, PDGFβ and Ang-2 (Gleadle and Ratcliffe 1998). In contrast, Rac1 is shown to be involved in hypoxia-induced PI3K activation of HIF-1α through a different mechanism (Hirota and Semenza 2001). Hypoxia-induced expression of Rac1 also contributes to the upregulation of HIF-1α and, subsequently, VEGF in gastric and hepatocellular cancer cells (Xue, Bi et al. 2004). VEGF has been reported to increase RhoA activity and localization to the cell membrane, and the RhoA /ROCK pathway has been implicated in the VEGF-mediated angiogenesis (van Nieuw Amerongen, Koolwijk et al. 2003). In addition, RhoA activation also increases tyrosine phosphorylation of the primary VEGF receptor, VEGFR-2 (Gingras, Lamy et al. 2000).

Overexpression of RhoC in human mammary epithelial cells (HME) and a highly aggressive breast cancer cell line, SUM-149, increases VEGF expression (van Golen, Wu et al. 2000). Similar finding were found in the MCF10A cells (Wu, Wu et al. 2004), further suggesting that RhoC plays a role in, further suggesting that RhoC plays a role in increasing VEGF in mammary neoplasis.

5.5.2 IL-6 and IL-8 expression

IL-6 is a multifunctional cytokine that is involved in many different biological process, including immunological and inflammatory processes, tumor growth and angiogenesis (Hirano, Akira et al. 1990; Mateo, Reichner et al. 1994). IL-8 is another important cytokine that acts as a pro-angiogenic factor. Both of these cytokines can be induced by hypoxia (Yan, Tritto et al. 1995; Mizukami, Jo et al. 2005) and have been shown to upregulate VEGF mRNA expression (Cohen, Nahari et al. 1996). Studies indicate that active Rho proteins upregulate the expression of NFκB components in NIH-3T3 cells (Perona, Montaner et al. 1997; Montaner, Perona et al. 1998). Consistent with Rho-mediated activation of NFκB, HKG-CoA reductase inhibitors had been reported to reduce IL-6 expression by inhibiting Rho proteins (Ito, Ikeda et al. 2002). Rac1 has been shown to mediate the activation of a potential oncogen, STAT3, through NFκB regulated IL-6 signaling (Faruqi, Gomez et al. 2001).

IL-8 expression has also been found to be regulated by Rho proteins. In human endothelial cells, it has been shown that inhibition of RhoA, Rac1 and Cdc42 decreases NFκB activation and, therefore, decreases IL-8 mRNA and IL-8 protein expression (Hippenstiel, Soeth et al. 2000; Warny, Keates et al. 2000). In addition, RhoC has been shown to increase IL-6 and IL-8 expression in aggressive breast cancer cell lines (Xue, Bi et al. 2004). These evidences suggest that different Rho proteins modulate IL-6 and IL-8 through distinct signaling pathways.

5.5.3 FGF activation

FGF1 and FGF2 are the two earliest characterized members of the FGF family of growth factors. FGF is an angiogenic factor that is frequently overexpressed in breast and prostate cancers. Rac1 and Cdc42 have been reported to increase FGF1 expression by stimulating the FGF1 gene promoter region (Chotani, Touhalisky et al. 2000). One study demonstrated that Rac1 activity is required for FGF2-induced activation of Ras/MAPK signaling in human breast cell line MCF7 (Liu, Chevet et al. 1999). In addition, medium collected from RhoC stably transfected HME and SUM149 cells present higher level of FGF2, in comparison to those collected from control transfected HME cells (van Golen, Wu et al. 2000). However, how Rho proteins regulate FGF expression remains unclear.

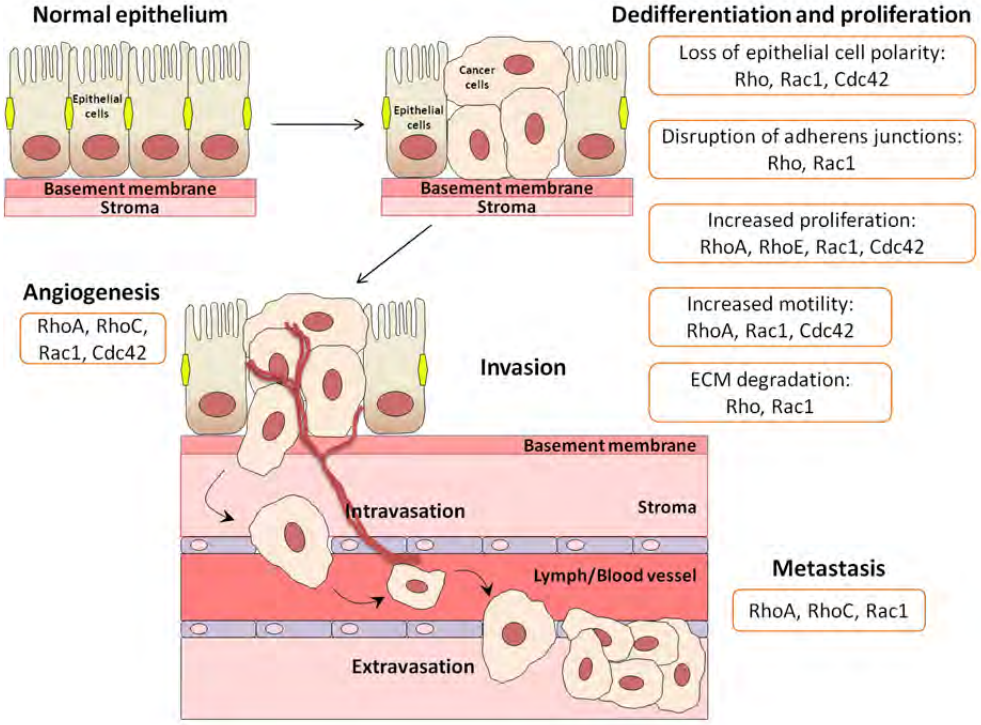

Fig. 4. Rho family GTPases are involved in different stages of breast cancer progression: dedifferentiation and upregulation of uncontrolled proliferation, angiogenesis, invasion and metastasis.

5.5.4 Repression of Tsp-1

The anti-angiogenic molecule Tsp-1 is capable of inhibiting metalloproteinase-9 (MMP9) from releasing the VEGF sequestered in ECM. The oncoprotein Ras has been reported to increase VEGF expression and inhibit Tsp-1 expression. One study showed that the inhibitory function of Ras on Tsp-1 via PI3K pathway also involve RhoA and RhoC in human embryonic kidney cell lines, human mammary cell lines, and breast cancer cell lines

(Watnick, Cheng et al. 2003). And the suppression of Tsp-1 always correlates with promotion of angiogenesis.

6. Conclusion

It is apparent that individual members of Rho GTPases play specific roles in different aspects in breast cancer development (Fig. 4). Aberrant expression and activity of Rho proteins contribute to the transformation from normal epithelial phenotype, increases in proliferation, the promotion of angiogenesis, elevated motility, and metastasis to distant organs. RhoA, RhoC and Rac1 are frequently overexpressed in metastatic breast cancers. Manipulating the Rho GTPases' regulatory proteins and their effectors can induce activation of Rho proteins, , leading to aberrant transcription factor activation, including that of NFκB, that contribute to invasive phenotypes. All this evidence suggests that Rho GTPases could be targets in cancer therapy. Therefore, better knowledge of the the regulation mechanisms of Rho GTPases in breast cancer may be critical for a more in-depth understanding of tumor biology, facilitating development of novel approaches for cancer treatment.

7. References

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